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MEIOTIC MATURATION AND THE CORTICAL GRANULE REACTION IN STARFISH EGGS

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Abstract

Correlative light and electron microscopic studies of immature and maturing starfish (*Asterias forbesi*) eggs have been carried out demonstrating (1) morphological alterations attending meiotic maturation induced by 1-methyladenine and (2) the structure of the egg cortex and cortical granule reaction. Because cortical granule components, are structurally recognizable, their fate and relation to the development of the fertilization membrane could be determined. One and possibly more of the cortical granule components become an integral part of the fertilization membrane. Comparison of maturing and immature ova indicate that germinal vesicle-containing oocytes (immature) are capable of undergoing a cortical granule reaction morphologically similar to that of eggs having undergone germinal vesicle breakdown (maturing).

Introduction

In the starfish, spawning and oocyte maturation are stimulated by the ovarian hormone, 1-methyladenine (1-MA), which is synthesized by follicle cells (Kanatani *et al.*, 1969). Isolated oocytes undergo germinal vesicle breakdown, shedding of follicular cells, and maturation in response to externally applied 1-MA at micromolar concentrations. Furthermore, application of 1-MA promotes uniform and synchronous maturation, thereby facilitating the study of oocyte maturation, fertilization, and early development. Ultrastructural investigations of starfish oocytes have examined oocyte-follicle cell relationships and surface changes stimulated by 1-MA (Hirai *et al.*, 1971; Rosenberg *et al.*, Schroeder *et al.*, 1979). As far as we are aware, correlative light and electron microscopic studies of germinal vesicle breakdown and meiotic maturation in *Asterias* oocytes treated with 1-MA have not been presented.

In addition, although the fine structure of the cortex of fertilized *Asterias* eggs has been examined (*cf.* Monroy, 1965), ultrastructural analysis of the cortical granule reaction in this organism has not been presented. Stages before, during, and after the cortical granule reaction in the starfish, *Patiria miniata*, have been described (Holland, 1980). In this study Holland (1980) discussed the question of the presence of a hyaline layer in activated starfish oocytes and suggested that observations made with *Patiria* are representative of the cortical granule reaction in other asteroids. The descriptions of the cortical granule reaction in *Asterias* presented herein have been carried out in light of Holland's speculations.

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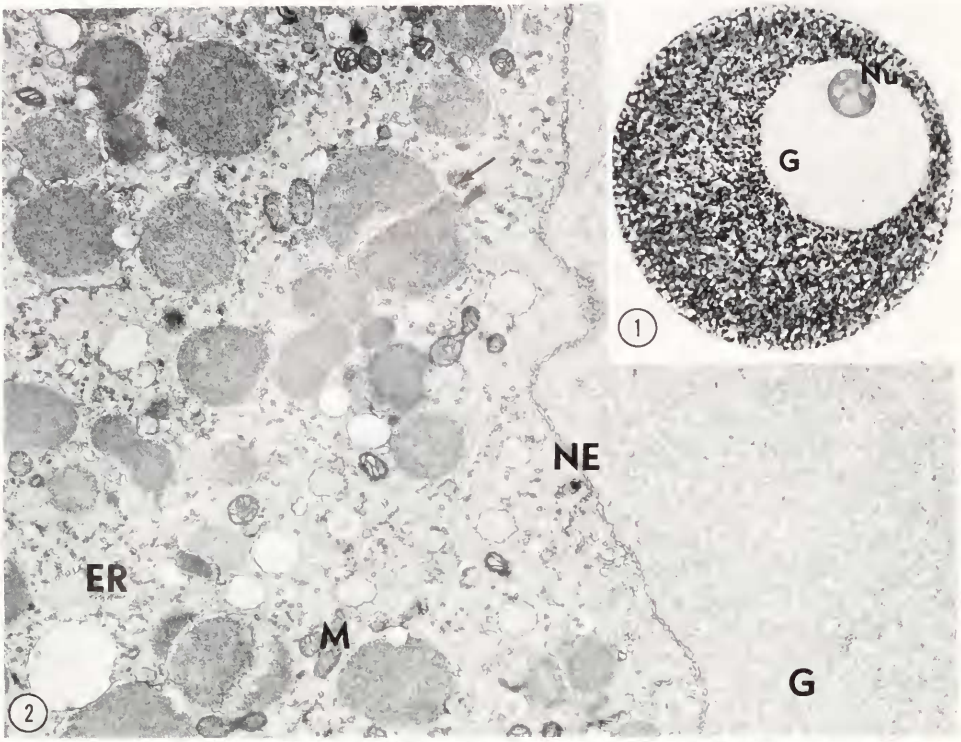
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Materials and Methods

Germinal vesicle-intact oocytes were obtained from ripe starfish (*Asterias forbesi*) ovaries which had been washed previously in calcium-free sea water (CaFSW; Schuetz and Biggers, 1968). Washing in CaFSW inhibited spontaneous nuclear maturation and induced the detachment of follicle cells from the oocytes. Ovaries were minced and germinal vesicle-intact oocytes were separated from follicle cells and returned to artificial sea water containing the normal concentration of calcium (MBL formula; Cloud and Schuetz, 1973). Germinal vesicle breakdown was induced by adding 1-MA (1 μg/ml; Sigma) to an oocyte suspension, and samples were taken at regular intervals and fixed for 1 hour at 4°C in a solution of sea water containing 2% gluteraldehyde, 0.5% paraformaldehyde, 1% acrolein, 1% sodium citrate, and 4.5% sucrose. The samples were washed overnight in sea water, incubated in 0.5% OsO_4 for 30 minutes, dehydrated in ethanol, and embedded in Spurr embedding medium. Manipulation of the specimens during these procedures has been described (Longo and Anderson, 1972). Thick sections were stained with 1% toludine blue and analyzed with a Leitz Orthoplan microscope. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

In order to examine the cortical granule reaction, germinal vesicle-intact eggs, induced to mature to the first metaphase of meiosis with 1-MA, and ova collected from spontaneously ovulating females which had undergone germinal vesicle breakdown were inseminated with sperm collected from isolated testes. Just prior to insemination sperm were diluted to 0.1% (v/v) in sea water. Specimens were fixed for 1 hour at 30-second intervals and then at 1-minute intervals for up to 10 minutes post insemination. Further processing was carried out as described above. In this report oocytes containing a germinal vesicle are referred to as "GV-intact" or "immature" ova; oocytes that have undergone germinal vesicle breakdown are referred to as "maturing" eggs.

Results

Germinal vesicle oocytes

The germinal vesicle of the *Asterias* oocyte was a large spheroid body, containing a homogeneous nucleoplasm in which was suspended a single nucleolus (Fig. 1). The nucleolus was composed of a dense, fine-textured material containing one or more areas of lesser density. The periphery of the germinal vesicle was delinated by a smooth-surfaced nuclear envelope (Fig. 2). Spheroid yolk bodies, containing a dense homogenous substance, were found in close association with one another; in these instances their juxtaposed surfaces were flattened. Vesicles, some comparable in size to yolk bodies but not nearly as numerous, were observed within the cytoplasm. In addition, smaller vesicles, some with a filamentous material, others lacking

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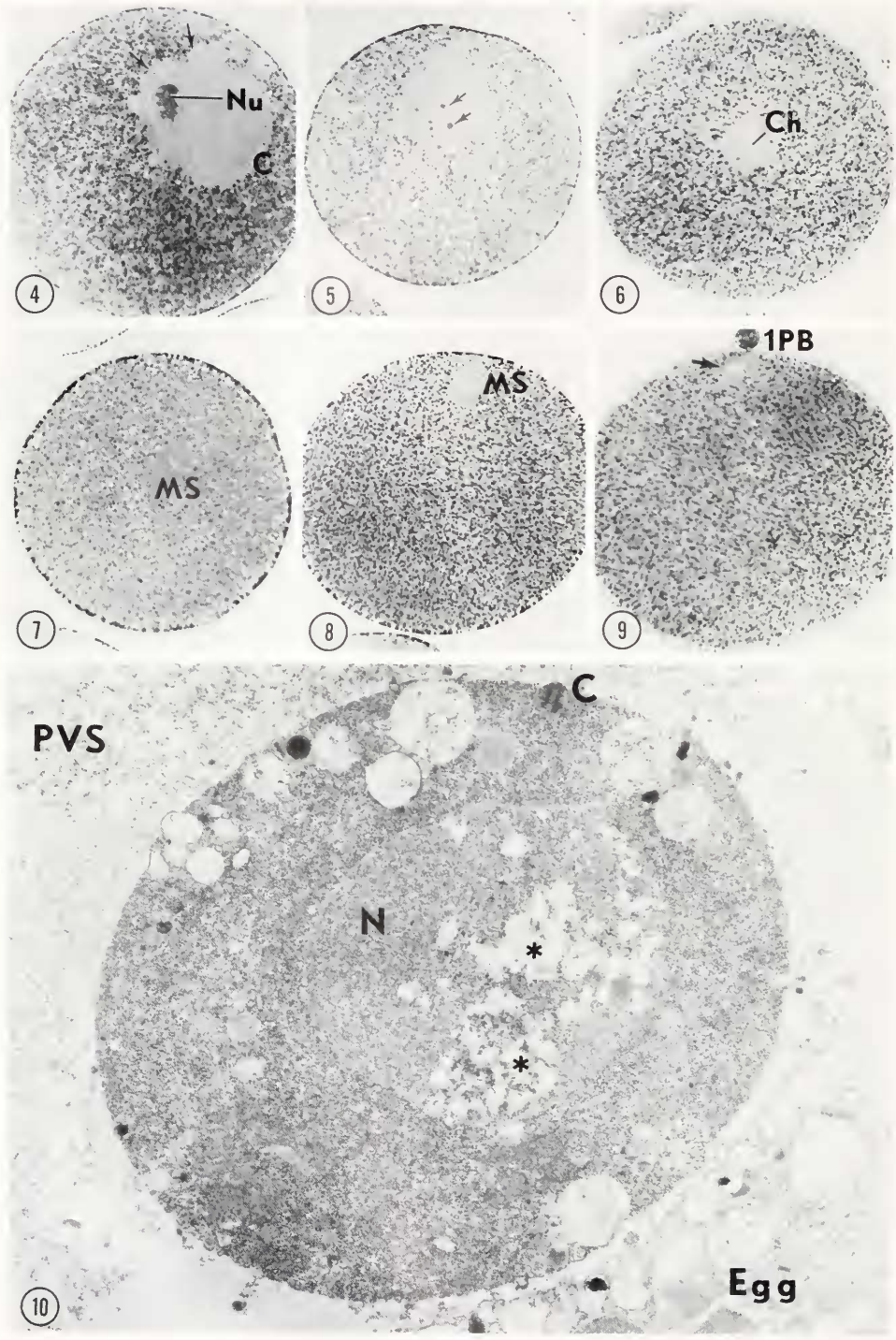
FIGURE 1. *Asterias* oocyte containing a germinal vesicle (G) and nucleolus (Nu) consisting of two structural components. The granularity of the cytoplasm is due to yolk bodies and mitochondria. ×1150.

FIGURE 2. Electron micrograph depicting a portion of the germinal vesicle (G) and adjacent cytoplasm. The yolk bodies are frequently found as aggregates seen at the arrow. M, mitochondria; ER, endoplasmic reticulum; NE, nuclear envelope. ×15,000.

FIGURE 3. Portion of a germinal vesicle initiating the resumption of meiosis (*i.e.*, germinal vesicle breakdown), 30 minutes after exposure to 1-MA. The vesicles depicted by the arrows are presumably products of the vesiculation of the nuclear envelope. Nu, disrupting nucleolus. ×10,000.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a substructure, were also present (Fig. 2). Small cisternae of endoplasmic reticulum, mostly of the smooth variety, as well as mitochondra were distributed throughout the cytoplasm. Golgi complexes were not prominent.

Germinal vesicle breakdown

Breakdown of the germinal vesicle in oocytes matured naturally or with exogeneous 1-MA was morphologically similar; the observations provided below are taken from studies where maturation was initiated by exogenous 1-MA. Furthermore, the application of 1-MA to immature oocytes allowed for a precise timing of meiotic maturation. Hence, the times referred to herein are based on counts of eggs where greater than 50% demonstrated a given stage of development ($N > 100$) and where the moment of addition of 1-MA was time-zero.

One of the earliest signs of germinal vesicle breakdown was the modification of the periphery of the germinal vesicle; *i.e.*, its surface became convoluted by 15 minutes after the addition of 1-MA, and this was followed by the disruption of the nuclear envelope (Figs. 3, 4). The nuclear envelope vesicutated, such that numerous vesicles were found along the interface of cytoplasm and the nucleoplasm (Fig. 3). Concomitantly, the nucleolus assumed a highly irregular profile and dispersed (Fig. 4). Continued meiotic maturation led to a considerable reduction in the volume formerly occupied by the germinal vesicle (Figs. 5–7). At 30 minutes after the addition of 1-MA the nuclear envelope and much of the nucleolus had disappeared. By 40 minutes after the addition of 1-MA the condensing chromosomes were apparent as "clear" areas rather than the usual opaque structures obtained in fixed preparations of other cell types (Fig. 6). Evidently, the preparative methods employed in this study removed portions of the chromosomes.

Within 60 minutes following the application of 1-MA, the chromosomes were observed associated with the forming meiotic spindle which was usually located in the central portion of the egg (Figs. 6, 7). Relative to the size of the egg the meiotic apparatus was small, measuring about 10 μm in length. It lacked prominent asters and appeared "barrel-shaped" when sectioned longitudinally. The spindle migrated to the animal pole of the egg and underwent its meiotic divisions (Figs. 8, 9).

The second polar body of a fertilized starfish egg is shown in Figure 10, 120 minutes after addition of 1-MA. The chromosomes taken into the second polar

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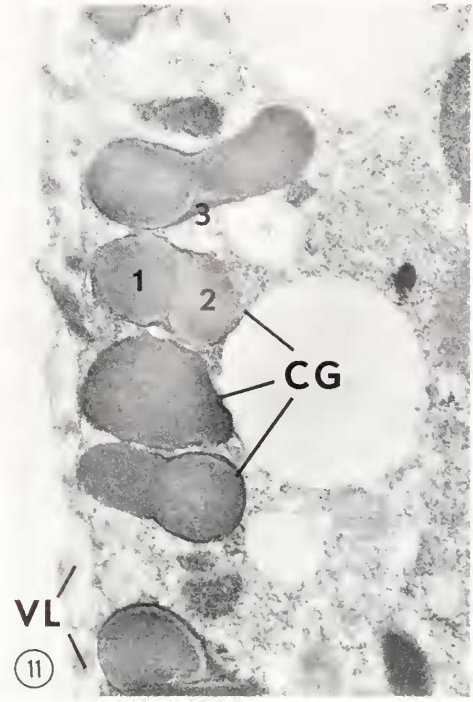
FIGURES 4–6. *Asterias* oocytes in successive stages of meiotic maturation. Eggs fixed at 15 (Fig. 4), 30 (Fig. 5), and 40 minutes (Fig. 6) after the addition of 1-MA. ×1150. Figure 4 depicts the development of plications along the periphery of the germinal vesicle (arrows), the disruption of the nucleolus (Nu), and the development of a "clearing" (C) of organelles along the periphery of the germinal vesicle. This is followed by the disappearance of the nuclear envelope (Fig. 5) and the condensation of the meiotic chromosomes (Ch), which are the lightly stained structures shown in the center of the egg in Figure 6. The structures depicted by arrows in Figure 5 are remnants of the nucleolus.

FIGURES 7 AND 8. Condensed chromosomes (arrows) organized on the meiotic spindle (MS) which is formed in the center of the egg (Fig. 7) and then moves to the cortex (Fig. 8). Specimens prepared at 60 and 70 minutes after addition of 1-MA, respectively.

FIGURE 9. *Asterias* egg having completed the formation of the first polar body (1PB); specimen fertilized after germinal vesicle breakdown. The chromosomes remaining within the egg are shown at the arrow, prior to their organization on the second meiotic spindle. ×1150.

FIGURE 10. Second polar body of a fertilized *Asterias* egg located within the perivitelline space (PVS) and containing a nucleus (N) and centriole (C). The areas within the nucleus indicated (*) represent chromatin which is dissolved by the preparative methods employed. × 36,000.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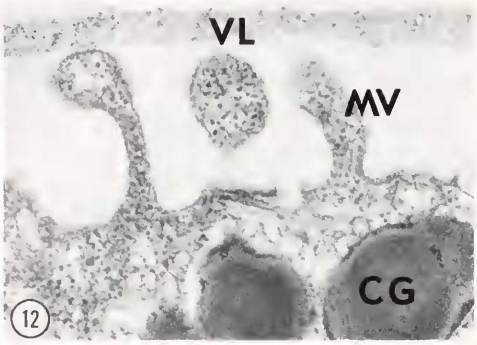
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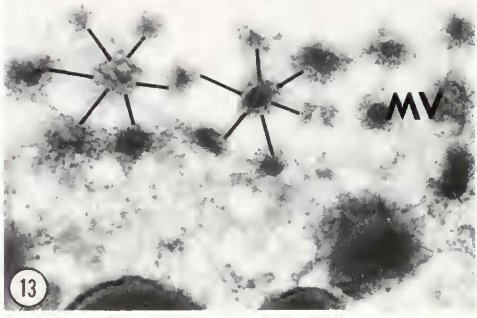
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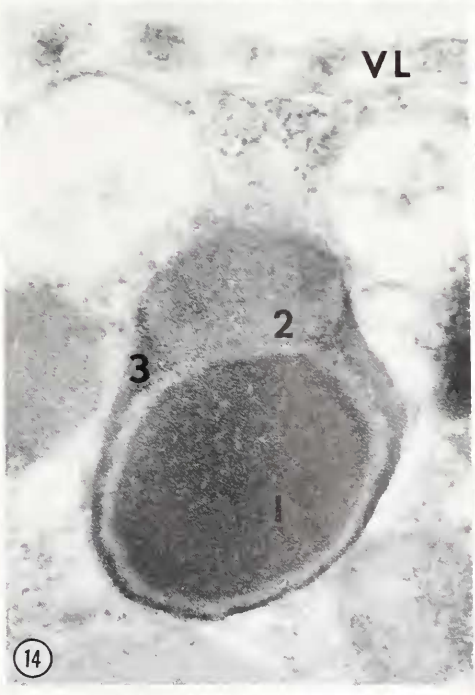
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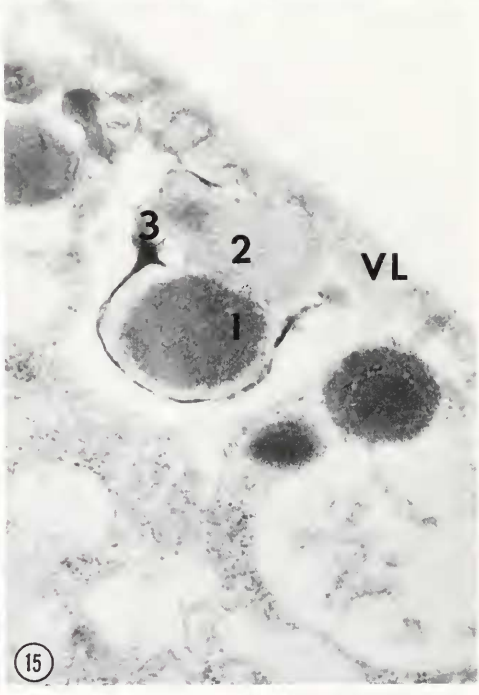
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body comprised a miniature nucleus. In addition to mitochondria and some vesicular structures, at least one centriole was observed within the second polar body.

Cortical granule reaction

The cortex of immature and maturing (from both 1-MA treated specimens and spontaneously ovulating females) oocytes appeared morphologically similar to one another. The plasma membrane was projected into numerous microvilli that were arranged in a hexagonal pattern and were covered by a prominent vitelline layer, composed of a filamentous material (Figs. 11–13). Although in some specimens the vitelline layer was separated slightly from the surface of the egg and only covered the tips of the microvilli (Fig. 12), it was morphologically similar to those that surrounded the microvilli (Figs. 11, 13). The separation of the vitelline layer was a random occurrence, seemingly unrelated to 1-MA treatment; its basis was not established. A monolayer of ellipsoid cortical granules was located within the cortex (Figs. 11, 12). The long axis of the granules was positioned at a right angle to the surface of the egg and measured 2 to 2.5 μm in length (Fig. 11). Structurally the granules usually contained three components that were resolved at high magnification (Fig. 14). The first was a spheroid mass of dense material, having a fine-textured appearance, which was positioned in the distal and/or proximal portions of the granule. Second, was a fine granular material of lesser electron opacity that often surrounded the first and filled much of the remainder of the granule. The third component was dense, relatively sparse in comparison to the other two components, and usually confined to the lateral aspect of the granule. In addition to the cortical granules some vesicular elements and mitochondria were present in the cortex of maturing and immature oocytes (Figs. 11, 14).

Insemination initiated the cortical granule reaction which was morphologically the same in immature and maturing eggs and comparable to that previously described for sea urchins (Anderson, 1968; Millonig, 1969). Consequently, only micrographs of the cortical granule reaction in maturing eggs are presented. Because of the three morphologically distinct components of the starfish cortical granule, their fate and relation to one another with respect to the formation of the fertilization membrane and organization within the perivitelline space could be followed. A dehiscing cortical granule is shown in Figure 15. Soon after fusion of the cortical granule membrane with the plasma membrane all three components of the cortical

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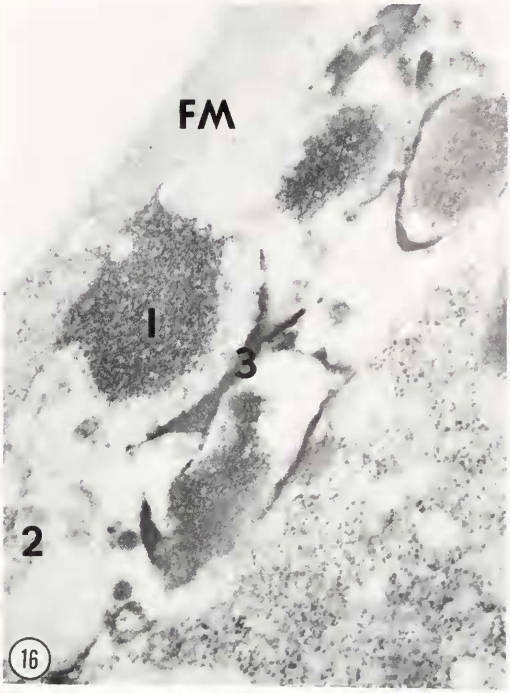
FIGURE 11. Cortex of an immature *Asterias* oocyte containing cortical granules (CG). The plasma membrane is covered by a vitelline layer (VL). Three structural components (1, 2, and 3; see text for explanation) may be seen in some cortical granules. × 27,000.

FIGURE 12. Portion of the cortex of an unfertilized maturing oocyte in which the vitelline layer (VL) has separated from the plasma membrane. Under these conditions the vitelline layer shows no changes in internal structure. MV, microvilli; CG, cortical granules. ×30,000.

FIGURE 13. Tangential section of the surface of a maturing *Asterias* egg demonstrating the organization of microvilli (MV). The filamentous material eminating from the microvilli represents a part of the vitelline layer. ×49,000.

FIGURE 14. Cortical granule of a maturing *Asterias* oocyte containing three structural components (1, 2, and 3; see text). A portion of the vitelline layer (VL) is depicted. ×33,000.

FIGURE 15. Dehiscing cortical granule of an inseminated, maturing oocyte. Initially, the more electron translucent component (2) is closely associated with the vitelline layer (VL) which is separating from the surface of the egg. Other components, which are still confined to the cup-like structure of the dehiscing cortical granule (1 and 3), are beginning to disperse. ×52,000.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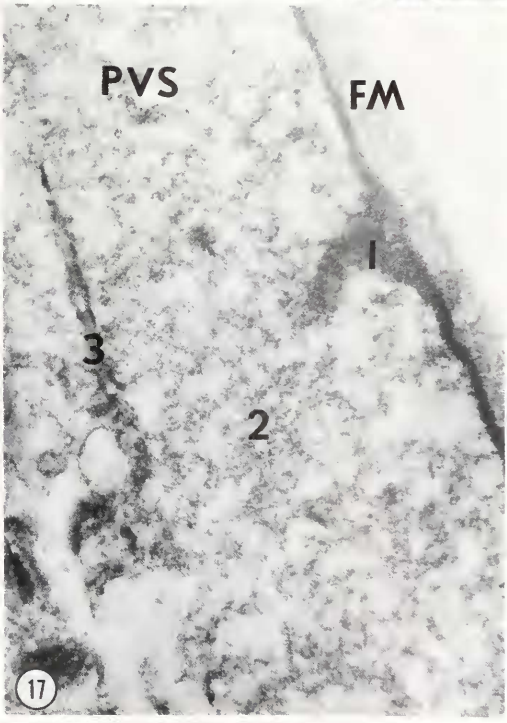
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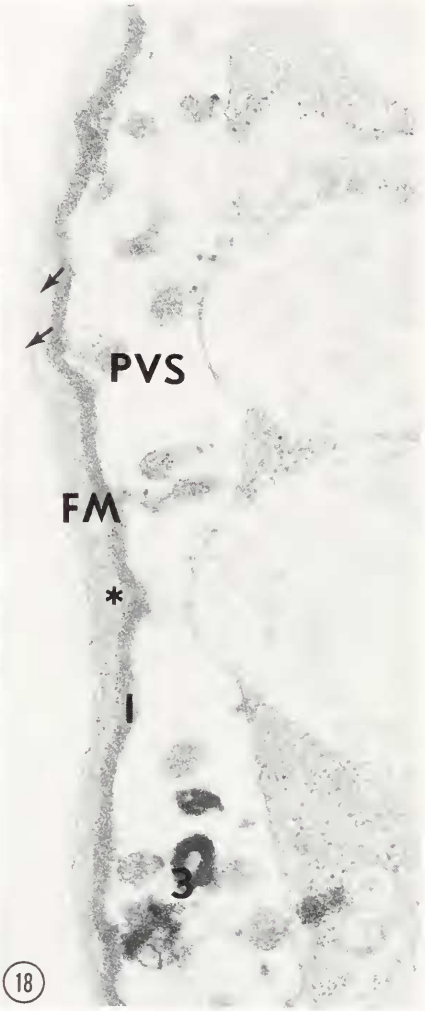
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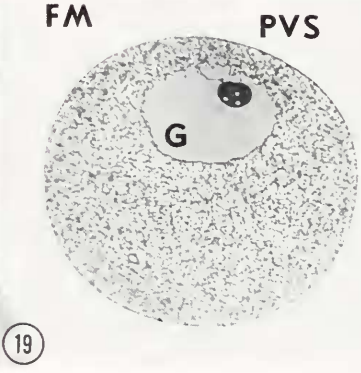
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granule appeared to swell. Initially the second component became associated with the vitelline layer. Later, however, much of this material appeared to disperse and fill the perivitelline space (Figs. 16, 17). The first component became associated with the vitelline layer; this material eventually coated the entire inner margin of the developing fertilization membrane and was seen as a dense layer (Figs. 16, 17). The fate of the third component was unclear; it appeared to form plate-like structures that were distributed throughout the perivitelline space (Figs. 16, 17).

Following the release of the cortical granule contents a well-defined fertilization membrane was formed in both immature and maturing ova (Fig. 18). Morphologically, it consisted of: (1) an outer laminated region apparently derived from the vitelline layer itself, with a possible contribution from the second component of the cortical granule, and (2) an electron opaque region along the innermost portion of the fertilization membrane consisting of material derived from the first component of the cortical granule (Fig. 18). The perivitelline space of both inseminated immature and maturing eggs was relatively large and measured up to 12 μm in width (Fig. 19); it was filled primarily with an electron translucent substance in which were found some dense structures apparently derived from the cortical granules.

Discussion

The observations of this study demonstrate: (1) morphological alterations and their chronology in *Asterias* eggs, induced by 1-MA, leading to the development of the second polar body, (2) the structure of the *Asterias* egg cortex and cortical granule reaction, and (3) that germinal vesicle-containing oocytes of *Asterias* are capable of undergoing a cortical granule reaction morphologically similar to that of eggs having undergone germinal vesicle breakdown.

Germinal vesicle breakdown and meiotic maturation

The germinal vesicle of *Asterias* oocytes is morphologically comparable to those observed in eggs of other organisms (Kessel, 1968; Millonig *et al.*, 1968; Longo and Anderson, 1970). That 1-MA had an effect on meiotic maturation was first indicated by the undulation of the nuclear envelope of the germinal vesicle and a disruption of the nucleolus. These changes are characteristic of germinal vesicle breakdown as

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FIGURE 16. Cortex of a maturing oocyte, in which the contents of the cortical granules fill the perivitelline space. The cortical granule component designated 2 is dispersed within the perivitelline space and may have become integrated into the developing fertilization membrane (FM). Component 1 is seen as an electron dense aggregate closely associated with the inner margin of the developing fertilization membrane. Electron dense component 3 is distributed within the perivitelline space. ×49,000.

FIGURE 17. Perivitelline space (PVS) of a fertilized, mature oocyte in which one of the structural components (1) of the cortical granules has lined the inner surface of the developing fertilization membrane. The material distributed throughout the perivitelline space may be derived from component 2. The dense material (3) may be derived from the third component of the cortical granules. ×44,000.

FIGURE 18. Structural organization of the fertilization membrane (FM) of an inseminated, immature oocyte. The fertilization membrane has collapsed and, consequently, is located in close proximity to the egg surface. The outermost aspect of the fertilization membrane (*) consists of laminated regions (arrows). The innermost aspect of the fertilization membrane is lined by electron dense material derived from component 1 of the cortical granules. The dense material located within the perivitelline space (PVS) may be derived from component 3 of the cortical granules. ×28,000.

FIGURE 19. Immature ooctye 2 hours after insemination possessing a fertilization membrane (FM) and prominent perivitelline space (PVS). G, germinal vesicle. ×1300.

observed in oocytes of other organisms where meiotic maturation is induced by other means (Merchant and Chang, 1971; Calarco *et al.*, 1972; *cf.* Longo, 1973; Sorenson, 1973). The significance of the tortuous outline developed by germinal vesicles induced to break down has not been established. Similar distortions in nuclear structure observed in other cells may be due to fluxes of materials into and out of the nucleus (Monroy, 1965). Changes in the germinal vesicle of starfish oocytes induced by 1-MA are believed to be brought about by the production of maturation promotion factor which is also responsible for subsequent maturation events (Kishimoto *et al.*, 1981).

With the exception of the development of asters, formation of the meiotic apparatus in *Asterias* is similar to that described for *Spisula* and *Tubifex* (Longo and Anderson, 1969, 1970; Shimizu, 1981a, b). The meiotic spindle was formed in the central portion of the oocyte and then moved to the cortex. The meiotic spindle of *Asterias* was structurally similar to that observed in mouse eggs in that it lacked well-developed asters and was barrel-shaped (Szollosi *et al.*, 1972). Due to the relatively large size of oocytes we were unable to verify the appearance and number of centrioles in the meiotic spindles of *Asterias*; however, the presence of at least one centriole in the second polar body indicates that these organelles are probably an integral part of the meiotic apparatus. Thus, the situation differs from that observed in mammals. Characteristically, the meiotic spindle of mammalian oocytes lacks centrioles (Szollosi, 1972; Szollosi *et al.*, 1972).

Cortical granule reaction

There has existed in the literature a question as to whether or not immature starfish oocytes are capable of a cortical granule reaction and the formation of a fertilization membrane (*cf.* Masui and Clarke, 1979). It has been generally believed that germinal vesicle breakdown was necessary before the starfish egg was capable of a cortical reaction (Hirai *et al.*, 1971; Hirai, 1976). However, immature eggs incubated in calcium-free sea water were able to inseminate and undergo a cortical granule reaction (Cayer *et al.*, 1975; Schuetz, 1975). The results presented herein support and amplify these observations at the ultrastructural level of observation and indicate that the cortical granule reaction in *Asterias* ova, with or without germinal vesicles, is structurally similar.

The cortical granule reaction in *Asterias* is morphologically similar to that described by Holland (1980) for *Patiria miniata*. Because of structurally recognizable cortical granule components, their fate and relation to development of the fertilization membrane can be traced. The present study shows that the dense component of the cortical granules coats the inner margin of the vitelline layer and becomes an integral part of the fertilization membrane. A similar process has also been described for sea urchins and *Patiria* (Anderson, 1968; *cf.* Ito, 1969; Inoué and Hardy, 1971; Holland, 1980).

Investigators working with the eggs of different organisms have shown that cortical granule components become a part of the vitelline layer and their interaction is related to characteristics the fertilization membrane acquires with its development, *e.g.*, hardening (Endo, 1961; Wolpert and Mercer, 1961; Bryan, 1970; Grey *et al.*, 1974; Chandler and Heuser, 1980; *cf.* Shapiro and Eddy, 1980; Schuel *et al.*, 1982). A similar interaction may also exist in *Asterias*. That the vitelline layer of *Asterias* showed no change in structure when separated from the surface of eggs not having undergone a cortical reaction suggests that cortical granule material is necessary for the progressive structuralization of the fertilization membrane.

Despite the release of the entire population of cortical granules and evidence from other echinoderms demonstrating that components of the hyaline layer are derived from cortical granules (Kane, 1970; Stephens and Kane, 1970; *cf.* Schuel, 1978), a well-defined hyaline layer was not obvious in fertilized eggs of *Asterias.* Although some of the cortical granule material is incorporated into the fertilization membrane, the fate of the remainder is in question. Some material is seen within the perivitelline space. However, it is much too sparse to form a prominent layer as found in many sea urchins. One reason for the absence of a layer may be due to the relatively larger perivitelline space, characteristic of fertilized *Asterias* eggs. The cortical granule contents may fill this space, resulting in a relatively diffuse distribution. Holland (1980, 1981) has questioned the presence of a hyaline layer in starfish as found in echinoids. As indicated by Hall and Vacquier (1982), participation by the hyaline layer does not appear to be greatly relevant to echinoderm morphogenesis, as this structure is seemingly found in only echinoids and ophiuroids (*cf.* also Holland, 1981). In starfish, the interaction of blastomeres alone without the aid of an extracellular layer seems to be sufficient for blastula formation (Dan-Sohkawa, 1976; Dan-Sohkawa and Fujisawa, 1980).

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